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β1 integrin adhesion enhances IL-6 mediated STAT3 signaling in Myeloma cells: Implications for Microenvironment Influence on Tumor Survival and Proliferation

Kenneth H. Shain, **Danielle N. Yarde**, **Mark B. Meads**, **Mei Huang**, **Richard Jove**, **Lori A. Hazlehurst**, and **William S. Dalton***
Experimental Therapeutics Program at the H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive Tampa, FL 33612

Abstract

The bone marrow microenvironmental components interleukin (IL)-6 and fibronectin (FN) individually influence the proliferation and survival of Multiple Myeloma (MM) cells; however, *in vivo* these effectors most likely work together. We examined signaling events, cell cycle progression, and levels of drug response in MM cells either adhered to FN via β1 integrins, stimulated with IL-6, or treated with the two combined. While G_1/S cell cycle arrest associated with FN adhesion was overcome when IL-6 as added, the cell adhesion mediated drug resistance (CAM-DR) was maintained in the presence of IL-6. Concomitant exposure of MM cells to IL-6 and FN adhesion revealed a dramatic increase in STAT3 phosphorylation, nuclear translocation and DNA-binding, as compared to either IL-6 or FN adhesion alone in four MM cell lines. Importantly, this increase in STAT3 activation correlated with a novel association between STAT3 and gp130 in cells adhered to FN prior to stimulation with IL-6, relative to non-adherent cells. Taken together, these results suggest a mechanism by which collaborative signaling by β1 integrin and gp130 confers an increased survival advantage to MM cells.

Keywords

Adhesion; β1 integrin; IL-6; STAT3; Multiple Myeloma; microenvironment

INTRODUCTION

Multiple Myeloma (MM) is a B cell malignancy characterized by the monoclonal expansion of plasma cells. Although numerous genetic alterations have been implicated in MM pathogenesis, it is hypothesized that the bone marrow (BM) microenvironment also contributes to MM cell pathogenesis (1–6). The BM microenvironment consists of a complex array of factors that promote cell growth and survival, and may contribute to minimal residual disease and the development of acquired drug resistance (7–14). These bone marrow factors can be divided into two categories: 1) soluble factors including cytokines, chemokines, and growth factors, and 2) physical factors that include extracellular matrix (ECM) glycoproteins and bone marrow stromal cells.

*Please address correspondence to: William S. Dalton Ph.D., M.D. Departments of Experimental Therapeutics and Oncologic Sciences, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, 12902 Magnolia Dr. Tampa, FL 33612, Tel: (813) 745-4361, Fax: (813) 745-4258, E-Mail: E-mail: Dalton@moffitt.usf.edu.

Interleukin (IL)-6, regarded as one of the most important cytokines in MM disease progression, promotes the proliferation and survival of MM cells through the activation of Janus kinase (Jak)/STAT3, Ras/ERK1/2, and PI3 kinase/Akt/PKB signaling pathways (15–19). Signal transduction follows binding of IL-6 to gp80/IL-6Rα and recruitment of gp130/CD130 (20). The subsequent formation of a hexameric signaling complex, containing two molecules each of IL-6, gp80, and gp130, induces dimerization of gp130 and receptor phosphorylation by constitutively bound Jak family tyrosine kinases (Jak1, Jak2, and Tyk2) (21,22). In regard to STAT3 signaling, phosphorylation of four c-terminal tyrosine residues in the gp130 subunit has been proposed to facilitate the recruitment and phosphorylation of STAT proteins. STAT phosphorylation promotes its dimerization, nuclear translocation, and DNA-binding, followed by the transactivation of genes involved in a number of cellular functions including proliferation, differentiation, and survival (15,18,20,23).

Of the physical factors of the BM microenvironment, the glycoprotein fibronectin (FN) has also been demonstrated to control the growth, survival, and drug resistance of MM cells (1,4, 9–12). FN adhesion activates α4 or α5 and β1 (α4β1 & α5β1) integrin heterodimers via unique molecular aggregates utilizing associated non-receptor protein tyrosine kinases (PTK) and in some cases recruitment of receptor protein tyrosine kinases (RPTKs) for intracellular signaling (24,25). Signaling via these integrin complexes confers resistance to a multitude of pro-apoptotic effectors including serum deprivation, chemotherapeutic drugs, and death receptor ligation (CD95/Fas) (13,26).

Because of the influence of the BM microenvironment in MM progression, it is important to define not only the role of individual components of the BM, but also the manner by which both soluble and physical factors of the microenvironment collaborate in MM tumorigenesis. To date, the soluble factor IL-6 and the physical factor FN have been examined independently. However, *in vivo* it is most likely that these factors collaborate to influence myeloma cell survival. Therefore, elucidation of biochemical mechanisms associated with these factors alone, and in combination, is important in defining the effects of the microenvironment on cell survival and proliferation.

In this report, utilizing a simple cellular model, we examined intracellular signaling and the biological sequelae following stimulation with IL-6 alone, FN adhesion alone, or their combination in MM cell lines. Importantly, we demonstrate that IL-6 and FN adhesion collaborate to activate STAT3, but not ERK1/2 or Akt. Furthermore, this collaboration parallels an increase in gp130 complex phosphorylation and a novel IL-6 independent pre-association of STAT3 with gp130 when cells are adhered to FN. This work is significant because it shows that IL-6 stimulation does not reverse CAM-DR, but does reverse FN-mediated cell-cycle arrest. Taken together, these results suggest a mechanism by which collaborative signaling by β1 integrin and gp130 confer a survival advantage to MM cells.

MATERIALS AND METHODS

Cells and Materials

Four MM cell lines utilized: RPMI 8226 (RPMI media supplemented with 5% heat inactivated FBS), MM1.S (kindly provided by Dr. Steve Rosen, Northwestern University, Chicago, Illinois; 10% FBS, RPMI media), H929 (10% FBS, RPMI media supplemented with 0.0004% β-mercaptoethanol), and U266 (10% FBS, RPMI media). Cells were maintained in culture in the respective concentration of FBS in RPMI media supplemented with 1.0% penicillin/ streptomycin and L-Glutamine (Gibco, Grand Island, NY). Experiments (up to 6 hours) were carried out under serum free conditions. Antibodies utilized: STAT3 (1:2000) and phosphor-STAT3 (1:1000) in 5% BSA/TBS 0.1% Tween 20 (Cell Signaling, San Diego, CA), gp130 (1:250; BD Pharmingen, San Diego, CA), β1 integrin (1:250; Chemicon, Temecula, CA), β-

actin (1:10,000; Sigma-Aldrich, St. Louis, MO). Recombinant human IL-6 was purchased from Sigma-Aldrich.

Protein isolation and Western Blot Analysis

Cells, following incubation under the indicated conditions, were washed twice with ice cold PBS, and incubated for 10 minutes at 4 °C in Triton X-100 lysis buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 25 mM NaF, 1% Triton X- 100, 10% glycerol, 2 mM Naorthovanadate), with 1 Complete, Mini protease inhibitor cocktail tablet (Roche, Mannheim, Germany) per 10 ml Triton X-100 buffer. Protein lysates were quantitated with Bio-Rad reagent (Bio-Rad, Hercules, CA) and 10–50 μg of cellular lysates were separated on 12.5% polyacrylamide gels and transferred to PVDF membrane. Protein levels were examined with specific antisera and visualized with SuperSignal-Pico or SuperSignal-Dura Light (Pierce, Rockford, Illinois).

EMSA Analysis

STAT3 DNA-binding assays were performed on nuclear extracts prepared in hypertonic buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, and 0.5mM PMSF). Five 5μg of total protein were incubated with a double-stranded ^{32}P-radiolabeled hSIE oligonucleotide probe and visualized as previously described (15).

Immunoprecipitation

Cells were maintained in suspension or adhered to FN for 60–90 minutes in presence or absence of 1.0 ng/ml rhIL-6. Cells were washed twice in ice cold PBS and harvested as previously described (1). Cells were incubated in Tris buffered saline (TBS: 30 mM Tris HCl (pH 7.4), 150mM NaCl,) containing 1% NP-40, 0.1% sodium azide, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, and 1 Complete, Mini protease inhibitor cocktail tablet for 10 minutes on ice. Cellular debris was removed by centrifugation at 10,000 × *g* for 10 minutes at 4°C. Lysates were quantified via Bio-Rad reagent and between 2 and 4 mg of protein were used per sample. Samples were pre-cleared with 10 μl of Protein Plus A/G agarose beads (Santa Cruz Biotech, Santa Cruz, CA) for 60 minutes rotating at 4°C. Slurries were centrifuged (800 × *g* for 1 minute at 4°C) and supernatant was transferred to fresh tubes containing 1μg anti-gp130 antibodies or isotype control antibodies (BD Pharmingen) and incubated overnight rotating at 4°C. Protein Plus A/G agarose beads (10μl) were added and lysates were incubated an additional 2 hours. Immunoprecipitates were subsequently washed twice in TBS containing 1% NP-40 and 0.1% sodium azide, and three additional times with TBS containing 0.1% sodium azide and 0.1% bovine serum albumin (Roche). Immunoprecipitates were mixed with 4X SDS loading dye and 100 mM DTT and incubated for 5 minutes at 95°C. Following centrifugation (800 × *g* for 2 minutes) samples were separated on 4–12% NuPAGE SDS-PAGE gels (Invitrogen).

MTT

All assays were performed on cells in logarithmic growth. Cytotoxicity was determined by MTT assay as previously described (1). Briefly, cells were incubated with serial dilutions of mitoxantrone or doxorubicin (Sigma, St. Louis, MO) under the various conditions for 96 hours. Cells were then incubated with 50 μl MTT dye (Sigma) for four hours. Plates were centrifuged, aspirated and formazan was solubilized with DMSO. Optical density was determined with a Dynex II ELISA plate reader at 540 nm (Dynex Technologies, Chantilly, VA).

BrdU Analysis

The effects of FN adhesion +/−IL-6 on cell cycle progression were measured by two-color flow cytometry using FITC-conjugated anti- bromodeoxyuridine (BrdU) antibody and PI staining (BD Biosciences, San Jose, CA) of DNA content. Cells were incubated on FN-coated

60 mm dishes or maintained in suspension on non-coated plates for 48 hours. Following the 48-hour incubation, samples were prepared as previously described (4). Following preparation, samples were analyzed for BrdU incorporation and PI staining by flow cytometry.

RESULTS

Adhesion of the RMPI 8226 MM cell line to FN enhances IL-6 signaling

Signaling by the soluble BM effector IL-6 elicits biological effects in MM cells via Jak/STAT3, ERK1/2, and PI3-K/Akt signaling pathways (15,16,19). Cell adhesion to FN via β1integrins also initiates several signaling pathways involving ERK1/2, Akt, src-family tyrosine kinases, and RelB (25,27). To date, the intracellular signaling of these two determinants has been examined independently. Here, we investigated the combination of both IL-6- and FN-mediated cell adhesion. As demonstrated in Figure 1A, adhesion of RPMI 8226 cells to FN enhanced their response to IL-6 stimulation. Treatment of FN-adhered RPMI 8226 cells with 0–10 ng/ml recombinant IL-6 results in a dose-dependent increase in the phosphorylation of STAT3 and ERK1/2, with no consistent stimulation of Akt, at two hours post-stimulation with IL-6 (Fig 1A; ERK and Akt data not shown). Further comparison of IL-6 dependent STAT3 phosphorylation in suspension and adhered cells revealed a 10-, 6.25-, and 3-fold increase in STAT3 hosphorylation in adhered cells over suspension cells at 0.1, 1.0, and 10ng/ml of recombinant IL-6, respectively. A parallel 3-fold increase in STAT3/DNA complexes was seen in cells adhered to FN relative to cells in suspension, as measured by electrophoretic mobility shift assay (Fig 1B). Super-shift assays demonstrated primarily STAT3 homodimers, with a paucity of STAT3: STAT1 heterodimers being observed (data not shown). These results demonstrate that adhesion of MM cells to FN alters STAT3 signaling via the IL-6 receptor. One explanation for the increased capacity of IL-6 signaling in RPMI 8226 cells adhered to FN may involve an up-regulation of the IL-6 receptor α (gp80) or gp130. Flow cytometric analysis demonstrated that surface expression of both gp80 and gp130 remained unchanged following adhesion of RPMI 8226 cells to FN (data not shown), indicating that the observed changes in STAT3 phosphorylation are not mediated by changes in the levels of IL-6 receptor components.

To examine the possibility that FN-adhesion stimulates IL-6 production in an autocrine fashion, we measured IL-6 production by ELISA in supernatants from RPMI 8226 myeloma cells cultured in suspension versus those adhered to FN. Under both conditions, soluble IL-6 remained undetectable from 0.5 to 4.0 hours (data not shown). Based on these findings we conclude that an FN-adhesion mediated production of IL-6 does not contribute to STAT3 signaling in co-stimulated cells.

FN adhesion results in a sustained activation of STAT3 phosphorylation and DNA binding

Signaling via cytokines and associated receptors occurs via a transient early activation of specific signaling determinants on the order of minutes. As demonstrated in Figure 2, co-stimulation of RPMI 8226 cells with IL-6 and FN augments STAT3 phosphorylation (30 minutes), and this increase in STAT3 phosphorylation is maintained for at least 6 hrs (Fig 2A). Mobility shift assays similarly demonstrated amplified and sustained STAT3 DNA-binding in cells adhered to FN following incubation with IL-6 as compared to cells maintained in suspension (Fig 2B). These results demonstrate that the augmentation of IL-6-mediated STAT3 signaling is maintained for up to 6 hours in RPMI 8226 cells adhered to FN relative to cells not engaged with FN.

The collaboration between β1 integrin-mediated adhesion and IL-6 is observed in all four MM cell lines examined

In this report, we have demonstrated that cellular adhesion to FN potentiated IL-6 stimulated STAT3 activity in the RPMI 8226 cell line. Similar results were observed in three additional MM cell lines. As shown in Figure 3, STAT3 phosphorylation in MM1.S, U266, and H929 MM cells was increased 1.9 ($p<0.05$), 4.1 ($p<0.05$), and 5.5-fold ($p<0.05$), respectively, in cells co-stimulated with IL-6 and adhesion to FN when compared to cells treated with IL-6 in suspension. Importantly, the increased ERK1/2 phosphorylation observed in the RPMI 8226 cell line following co-stimulation was not consistent across the cell lines (data not shown). Furthermore, although phosphorylation of Akt was observed in the MM1.S and H929 MM cell lines following IL-6 stimulation, this response was unchanged by FN-mediated cell adhesion (data not shown). Together, these results suggested that the effects of FN adhesion on IL-6 signaling may be relatively specific for STAT3.

Activation of β1 integrins further augments IL-6-mediated STAT3 activity

We have demonstrated that MM cell adhesion to FN augments IL-6 induced STAT3 signaling. However, the specific contribution of β1 integrins has yet to be addressed. Our lab has previously demonstrated that pre-treatment of RPMI 8226 cells with antibodies that block β1-integrins inhibits adhesion to FN (1). In the following experiments we utilized antibodies to β1 integrins that instead promote an active conformation, thereby increasing the binding potential of β1 integrins. To this end, pre-treatment of cells with β1 integrin activating antibody should enhance the amplification of IL-6-dependent STAT3 activation caused by FN adhesion, if β1 receptors are involved. The U266 MM cell line only modestly adhered to FN and pre-treatment of cells with β1 integrin-activating antibodies increased U266 cellular FN adhesion (data not shown). In Figure 4, we demonstrated that adhesion of U266 cells to FN enhances IL-6 mediated STAT3 protein phosphorylation and DNA-binding. Further, pre-incubation of U266 MM cells with activating β1 integrin antibodies further enhanced the effects of adhesion on STAT3 phosphorylation (Fig 4A), nuclear translocation, and STAT3 DNA binding (Fig 4B) in co-stimulated cells. These data demonstrate a central role for β1 integrin-mediated adhesion in the heightened response observed with the combination of IL-6 and FN adhesion on STAT3 signaling. Additionally, we demonstrate that pre-treatment with IL-6 blocking antibodies attenuates the signaling effects mediated by the collaboration between IL-6 and β1 integrin mediated adhesion. IL-6 receptor blocking antibodies also completely blocked the modest increase in STAT3 phosphorylation and DNA binding observed in U266 cells adhered to FN with β1 activating antibodies in the absence of exogenous IL-6 (Fig 4A & B, **lane 6 vs. lane 8**). These results indicate that the observed slight increase in STAT3 phosphorylation in the absence of recombinant IL-6 may result from the potentiation of relatively low levels of cytokine produced in the IL-6 autocrine loop known to be present in the U266 MM cell line (15). This slight increase, however, is very minor compared to the dramatic increase observed when these cells are co-stimulated with both IL-6 and β1 integrin mediated adhesion (Fig 4), suggesting that any autocrine IL-6 produced by U266 cells does not contribute substantially to the observed amplification of IL-6 dependent STAT3 activation mediated by adhesion to FN.

Adhesion of MM cells to FN facilitates an IL-6-independent recruitment of STAT3 to gp130

The signaling events initiated following IL-6 ligation are relatively well characterized. IL-6 binding to its cognate receptor facilitates gp130 homodimerization, activation of Jak family kinases, phosphorylation of specific tyrosine residues within the cytoplasmic domain of gp130, and activation of STAT3 (20). To further characterize the receptor proximal effects of FN adhesion on IL-6 signaling, we immunoprecipitated the IL-6R complex with antisera to gp130. Immunoprecipitation of gp130 revealed enhanced tyrosine phosphorylation of the gp130/Jak

family complexes following stimulation of FN-adhered RPMI 8226 cells with IL-6 (Fig 5 - phosphotyrosine at 125–130 kDa). Consistent with increased phosphorylation of the receptor complex, increased levels of phospho-STAT3 were found to be associated with gp130 under co-stimulatory conditions relative to IL-6 or FN adhesion alone. Interestingly, immunoprecipitation with gp130 antibodies also revealed an association between STAT3 (non-phosphorylated) and gp130 in the absence of IL-6 stimulation in cells adhered to FN (Fig 5, **lane 1 vs. 3**). To our knowledge, this is the first example of β1 integrin mediated *preloading* of STAT3 to the receptor complex. Moreover, these data suggest that FN adhesion facilitates an altered localization of STAT3, priming cells for signaling.

CAM-DR is maintained despite a reversal of FN-mediated cell cycle arrest following incubation with IL-6

In MM, IL-6 and FN adhesion have been demonstrated to protect cells from a host of cytotoxic stimuli (1,2,4,7,23). These reports suggest that the increased levels of STAT3 observed following IL-6 stimulation of FN-adhered cells may confer a greater protection against chemotherapeutics than FN adhesion alone. However, MTT cytotoxicity assays demonstrate that although adhesion to FN provides significant protection against treatment with either mitoxantrone or doxorubicin ($p<0.0002$ and $p<0.0001$, respectively), the addition of IL-6 provides no further protection (Fig 6A & B). Further analysis of drug-mediated apoptosis using FCM by Annexin-V/7-AAD corroborated these findings (data not shown).

Our laboratory has previously demonstrated that adhesion of the RPMI 8226 cell line to FN mediated a $p27^{Kip1}$ dependent G_0/G_1 cell cycle arrest.(4). As shown in Figure 6C, by BrdU/PI analysis, adhesion of 8226 cells to FN for 24 hours results in an increased number of cells in G_0/G_1 relative to cells maintained in suspension ($p=0.0028$), corroborating our previously published data. In contrast, when cells were adhered to FN in the presence of IL-6 no accumulation of cells in G_0/G_1 was observed, with levels similar to that observed in cells maintained in suspension with or without stimulation by IL-6 (Fig 6C). These results suggest that the addition of IL-6 bypasses or reverses the cell cycle arrest mediated by cellular adhesion to FN. Examination of $p27^{Kip1}$ expression demonstrated increased levels of $p27^{Kip1}$ protein following adhesion to FN relative to cells maintained in suspension, consistent with our original findings. Interestingly, although the combination of FN adhesion and IL-6 reversed/bypassed the cell cycle arrest mediated by adhesion alone, $p27^{Kip1}$ protein expression remained elevated under co-stimulatory conditions (data not shown). These data indicate that communication between integrins and IL-6 alters proliferative response mediated by either effector alone independent of $p27^{Kip1}$. Importantly, although the combination of IL-6 and FN adhesion does not further increase drug resistance compared to FN adhesion alone, the cell cycle arrest mediated by adhesion to FN is “reversed” by co-stimulation with IL-6. IL-6-induced cell proliferation may give adhered tumor cells an added survival advantage over the long term *in vivo*.

DISCUSSION

Research that focuses on the biology/pathogenesis of MM has long included the effectors of the bone marrow (BM) microenvironment. The soluble factor IL-6 and FN-mediated cell adhesion are two factors that independently control MM cell cycle progression and sensitivity to pro-apoptotic agents (1,4,9,10,12,14,23). However, the BM microenvironment likely affords MM cells a network of proliferative and survival factors; therefore, *in vivo* a MM cell receives stimulation from soluble and physical BM determinants simultaneously. In this report, using a *simple* model, consisting of only MM cell lines, IL-6 and FN, we begin to recapitulate this network of extracellular factors in order to investigate the molecular and biological consequences of co-stimulation of MM cells. We demonstrated that the combination of IL-6

and cellular adhesion to FN results in a dramatic amplification of STAT3 phosphorylation, nuclear translocation and DNA-binding via a novel *preloading* of gp130 with STAT3. These observations demonstrate collaboration between IL-6 receptor complexes and β1 integrins and underscore the complexity of interactions between MM cells and the BM microenvironment. Although interactions between integrins and cell surface receptors have been shown to enhance STAT activation (22,28–31), to our knowledge these results are the first illustrating specific STAT3 crosstalk between gp130 and integrins. Most previous reports demonstrating this phenomenon involved RPTKs (30,31); however, DeFilippi *et al* demonstrated a β1 integrin-mediated enhancement of STAT5 activation by the common IL-3 beta receptor (29). This receptor, like gp130, is a Type I cytokine receptor without intrinsic kinase activity (29). Here we identify a second Type I cytokine receptor involved in crosstalk between soluble and adherent factors, suggesting that this signaling mechanism involves a common mechanism across diverse receptor families. Furthermore, we describe a unique molecular mechanism by which cooperative signaling between gp130 and β1 integrins provides a survival advantage to MM cells treated with chemotherapeutic agents.

Stimulation of cells with IL-6 results in receptor multimerization and the activation of three major signaling pathways, the Jak/STAT3 pathway, the Ras/ERK1/2 pathway and PI3 kinase/Akt/PKB pathway (20). In this report, enhanced STAT3 activation was observed in all four MM cell lines examined. In contrast, enhanced ERK1/2 activation was observed in only 1 out of 4 cell lines, and, although Akt activation was observed in 3 of 4 cell lines, in all three cases it occurred independently of FN adhesion. These results indicate that the mechanism of collaboration is relatively specific for STAT3. Consistent with these observations, we demonstrated that the enhanced activation of STAT3 correlated with a FN adhesion-mediated binding of STAT3 to gp130.

Our observations suggest that FN adhesion facilitates a *preloading* of gp130 with STAT3 that enhances STAT3 activation. Dogma dictates that recruitment of STAT3 follows gp130 receptor stimulation and phosphorylation. So, in this context, our results suggest that either: 1) the adhesion of MM cells to FN facilitates the phosphorylation of gp130 and recruitment of STAT3 without measurable STAT3 activation - in agreement with current dogma, or 2) FN adhesion facilitates the association between gp130 and STAT3 independent of gp130 phosphorylation - suggesting a more complex regulation of STAT3 signaling from gp130. To address the former, integrin signaling from focal adhesions has been shown to involve ligand-independent activation of receptor protein tyrosine kinases (RPTKs) (24,32). To this end, the collaboration between β1 integrins and gp130 may involve a direct association and activation of gp130 by integrin ligation and focal adhesion formation. To date, we have been unable to demonstrate direct association between gp130 and β1 integrins (data not shown), but that does not fully exclude a direct or indirect activation by focal adhesion-associated tyrosine kinases.

It is also possible that we have identified an additional level of regulation. Although gp130 phosphorylation has been shown to be integral in STAT3 activation, the growing list of STAT chaperone proteins suggests that regulation of gp130/Jak/STAT signaling may be more complicated. The STAT chaperone proteins, including RACK1 (Receptor of Activated Protein Kinase C-1), GRP58/ER-60/ERp57 (Glucose Related Protein 58) and PKC-δ have been shown to enhance STAT signaling in several cellular models (33–35). One example, RACK1, a 36 kDa WD40-containing scaffold protein, has been found to participate in the enhanced activation of Src, PKC, STAT1 and most recently STAT3 (33,36,37). Zhang *et al* demonstrated that RACK1 facilitates the recruitment of STAT3 to Insulin and Insulin-like Growth Factor-1 (IR and IGF-1) receptors (33). Importantly, the effects of RACK1 were specific to STAT3, having no affect on Akt or ERK1/2 activation. Moreover, RACK1 has been shown to associate with β1 integrins and to facilitate formation of functional focal adhesions (38). These studies

suggest that RACK1 (or other chaperones) may be a catalyst for the collaboration between β1 integrin-mediated adhesion to FN and gp130.

Beyond the biochemical changes demonstrated in this report, the collaboration between IL-6 and FN cell adhesion also has biological consequences. Here we demonstrate that the collaboration of these two pro-survival effectors did not afford a survival advantage beyond levels conferred by CAM-DR. This was consistent with levels of Bcl-2, Bcl-X_L, and Mcl-1, which remained unchanged in response to co-stimulation in the RPMI 8226 cells (data not shown). In contrast, co-stimulation did facilitate an altered proliferative response. The collaboration between IL-6 and β1 integrins results in a bypass of the $p27^{Kip1}$ -dependent cell cycle arrest mediated by FN adhesion alone, in the face of unchanged levels of $p27^{Kip1}$ (4). Previous studies suggest that changes in cell cycle progression following enhanced STAT3 signaling involve increased levels of specific D-type cyclins (23). However, no observable increases in cyclin D expression were detected as a result of enhanced STAT3 activity in the RPMI 8226 cell line (data not shown). Therefore, it appears that β1 integrin and IL-6 mediated STAT3 signaling in RPMI 8226 cells may target alternate anti-apoptotic and/or proliferative factor(s).

Lastly, an expanding number of reports have been published examining the effects of bone marrow stromal cells on MM biology (reviewed in reference 14), providing novel insight into the dynamics between MM cells and the bone marrow microenvironment. In this report, we have “scaled back” the environmental cell model and used two relatively well-characterized MM bone marrow effectors to examine the biological consequences of co-stimulation in MM cells. We identify STAT3 as an important signaling effector involved in the collaboration between the physical environment and the soluble environment. Our findings suggest that adhesion to FN facilitates an alteration in the cellular localization of STAT3, *priming* gp130 for stimulation. As such, the environmental context of the cell may have significant bearing on intracellular signaling events, modulating both myeloma cell survival and proliferation.

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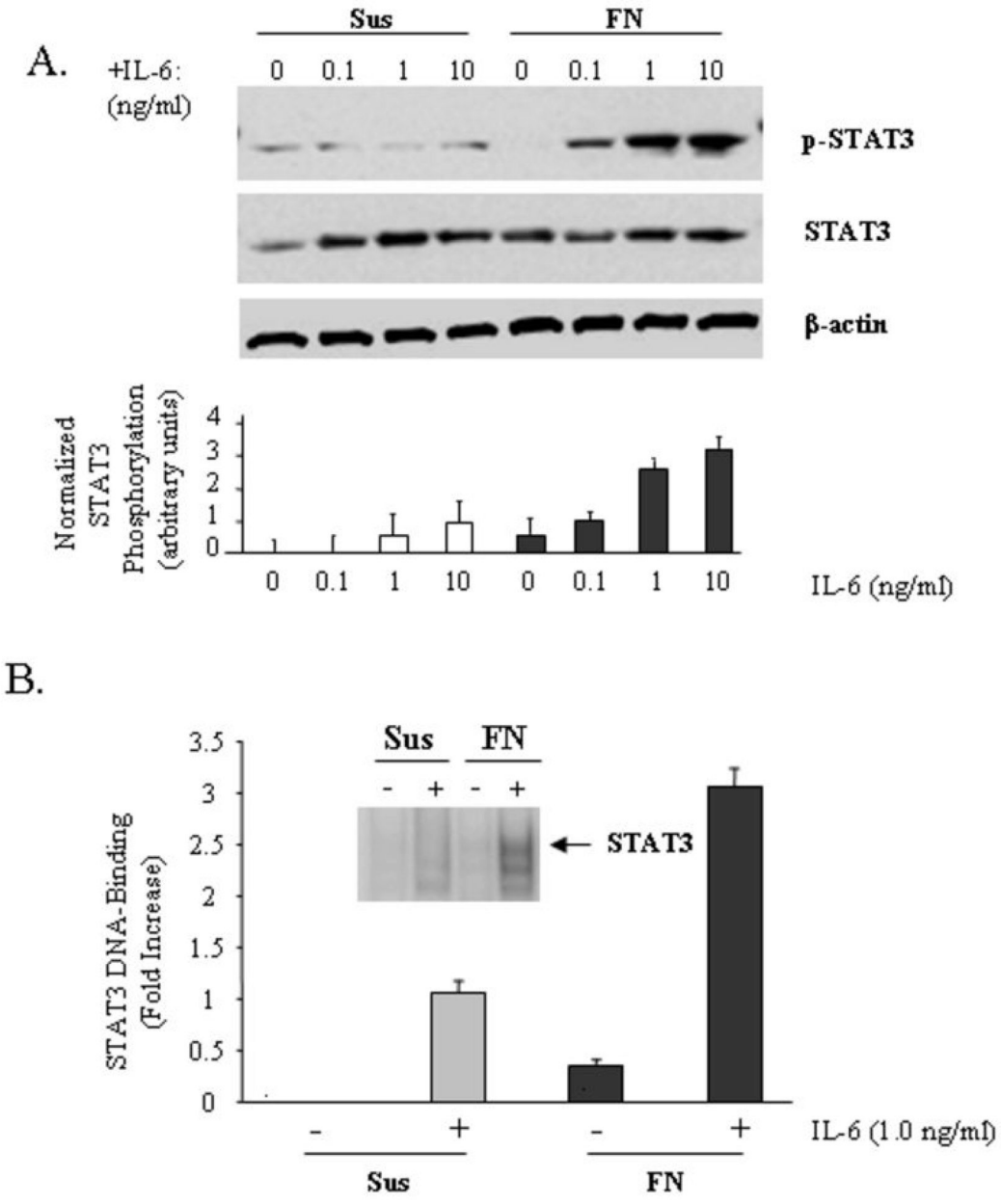

Figure 1. Adhesion of the RPMI 8226 MM cell line to FN augments IL-6-mediated phosphorylation, nuclear translocation, and DNA-binding

Cells were adhered to FN and incubated with the indicated dose of IL-6 for 2hrs. **A)** STAT3 phosphorylation is increased in a dose dependent manner in cells adhered to FN when compared to cells maintained in suspension (upper panel). Phosphorylated STAT3 levels were normalized to those of total STAT3 (ratio of phosphorylated/total STAT3 band intensities), demonstrating an amplification of IL-6 dependent STAT3 phosphorylation in cells adhered to FN compared to cells maintained in suspension (lower panel). Error bars represent standard deviations of the mean normalized STAT3 phosphorylation from three independent experiments. **B)** STAT3 nuclear translocation and DNA-binding are increased as measured by EMSA of nuclear lysates from RPMI 8226 cells adhered to FN or maintained in suspension plus or minus IL-6 (1.0ng/ml). Quantification of three independent EMSA assays demonstrated

a 3.0-fold increase in STAT3 DNA-binding in lysates from adherent cells incubated with IL-6, correlating with the phosphorylation data.

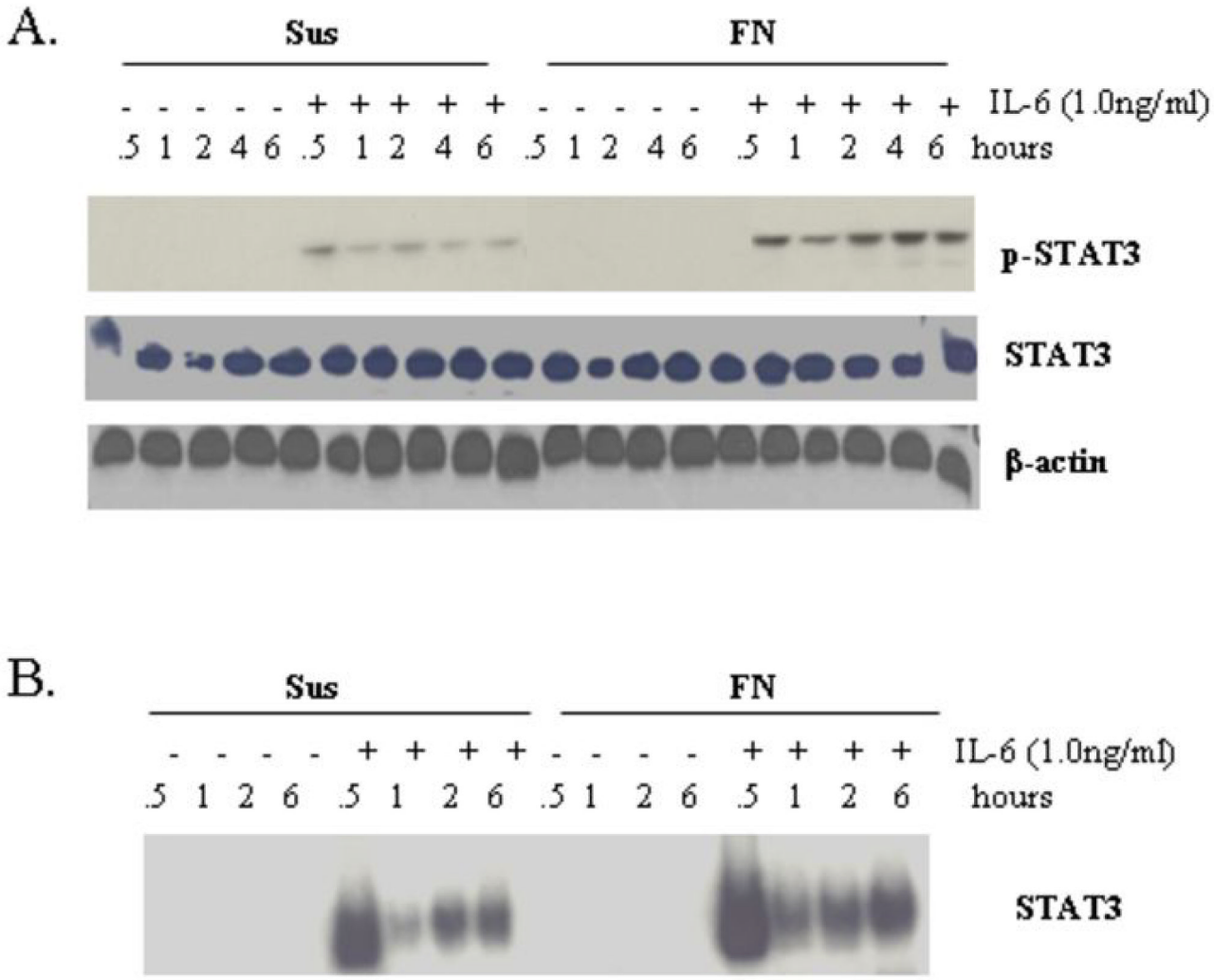

Figure 2. FN adhesion of the RPMI 8226 MM cell line augments IL-6-mediated STAT3 phosphorylation and DNA-binding for up to 6 hours

A) Western blot analysis demonstrated enhanced STAT3 phosphorylation 30 minutes following β1 integrin and IL-6 stimulation, and elevated levels of phosphorylated STAT3 remained for up to six hours in cells adhered to FN, as compared to cells maintained in suspension following incubation with 1.0 ng/ml IL-6. **B)** Increased STAT3 phosphorylation correlated with increased STAT3 nuclear translocation and DNA-binding as shown by EMSA.

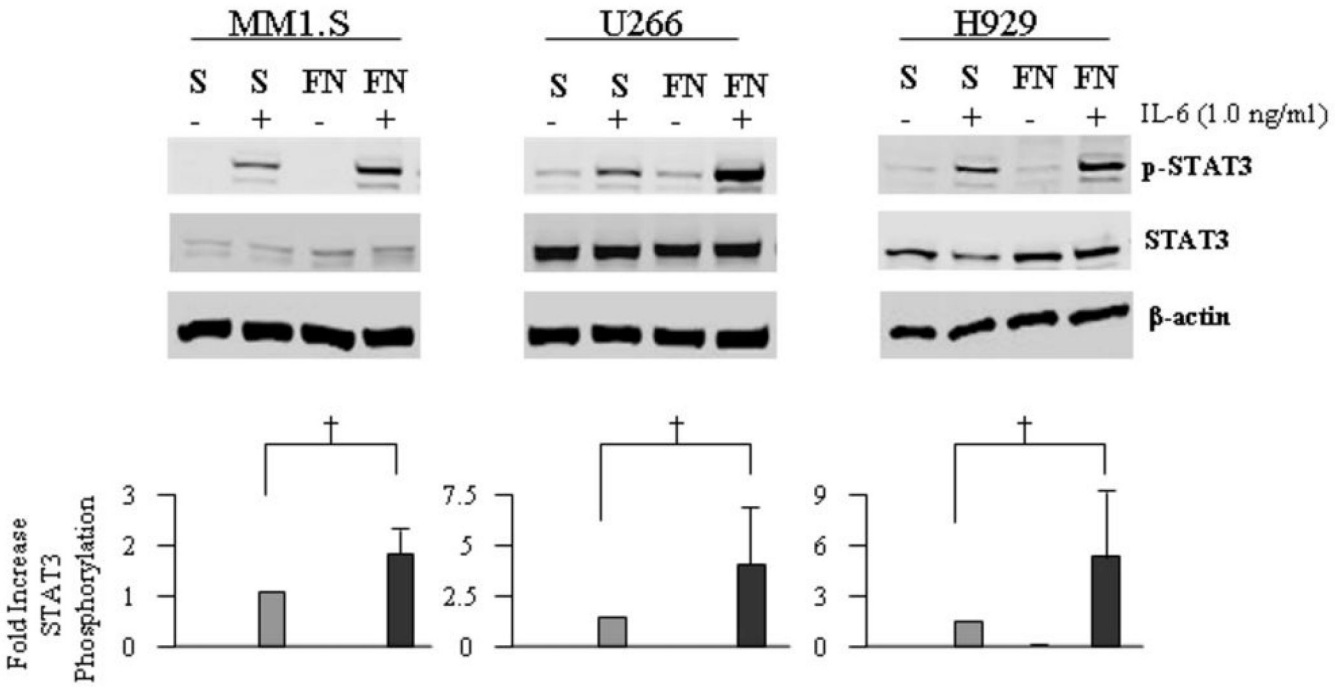

Figure 3. FN adhesion promotes increased IL-6-mediated STAT3 phosphorylation in MM1.S, H929, and U266 MM cell lines

Western blot analysis demonstrated increased STAT3 phosphorylation in MM1.S, H929, and U266 MM cells adhered to FN following stimulation with IL-6 (1.0ng/ml), relative to cells maintained in suspension or adhered cells alone. Quantification of three independent experiments (phosphorylated STAT3/total STAT3) using image quant software demonstrated that IL-6 dependent STAT3 phosphorylation in cells adhered to FN relative to those in suspension was increased: MM1.S- 1.87 fold (p=0.041); U266- 4.47 fold (p=0.027); H929- 5.47 fold (p=0.022).

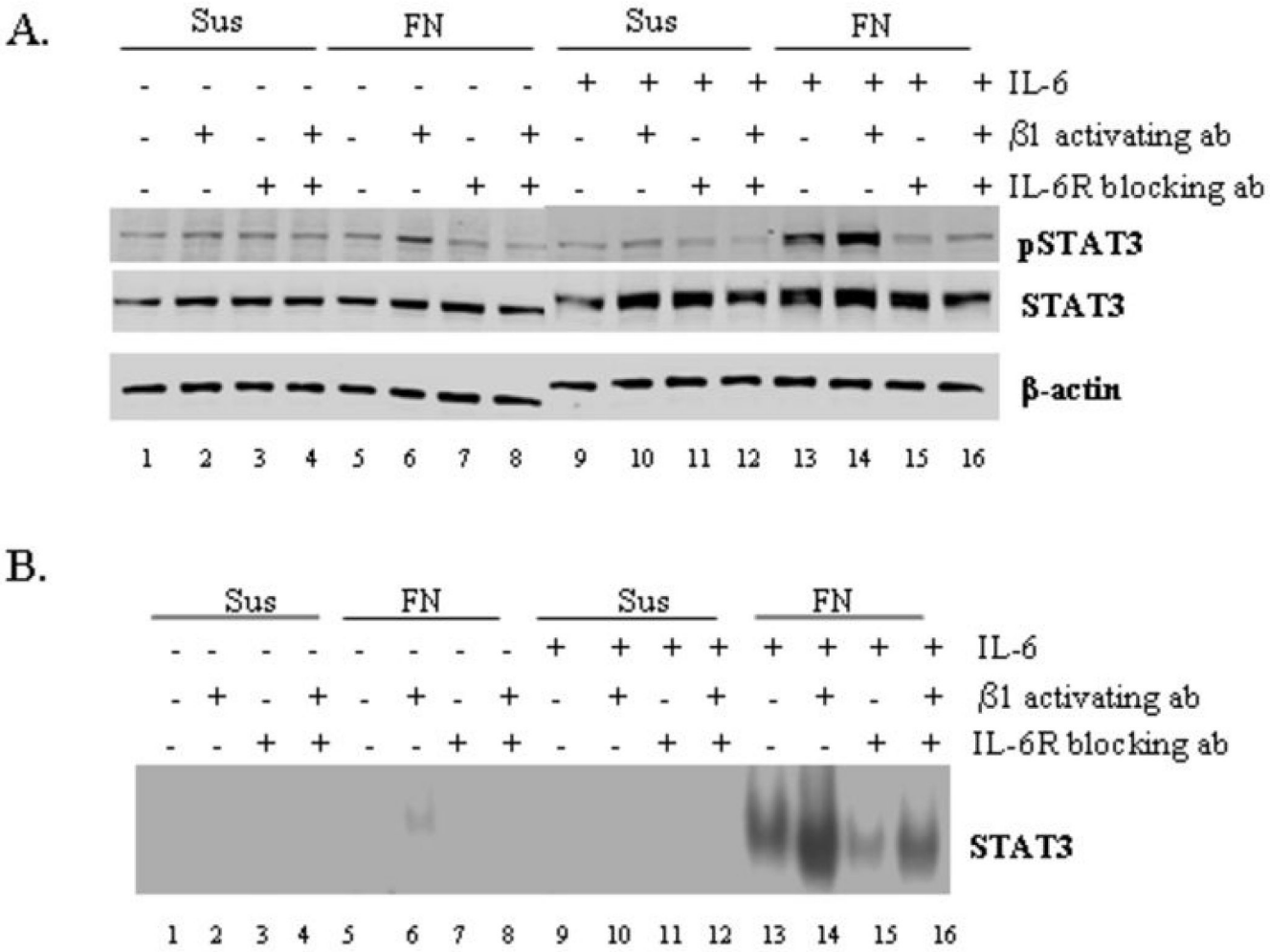

Figure 4. Enhanced β1 integrin mediated adhesion further augments STAT3 activity following IL-6 stimulation

β1 integrin-mediated adhesion of the U266 cell line to FN is enhanced by β1 integrin activating antibodies. Pre-treatment of U266 cells with β1 activating antibodies increases the STAT3 phosphorylation **(A)** and DNA-binding **(B)** mediated by IL-6 to a greater degree than FN alone. Further, pre-treatment of cells with IL-6 receptor blocking antibodies inhibited STAT3 activation.

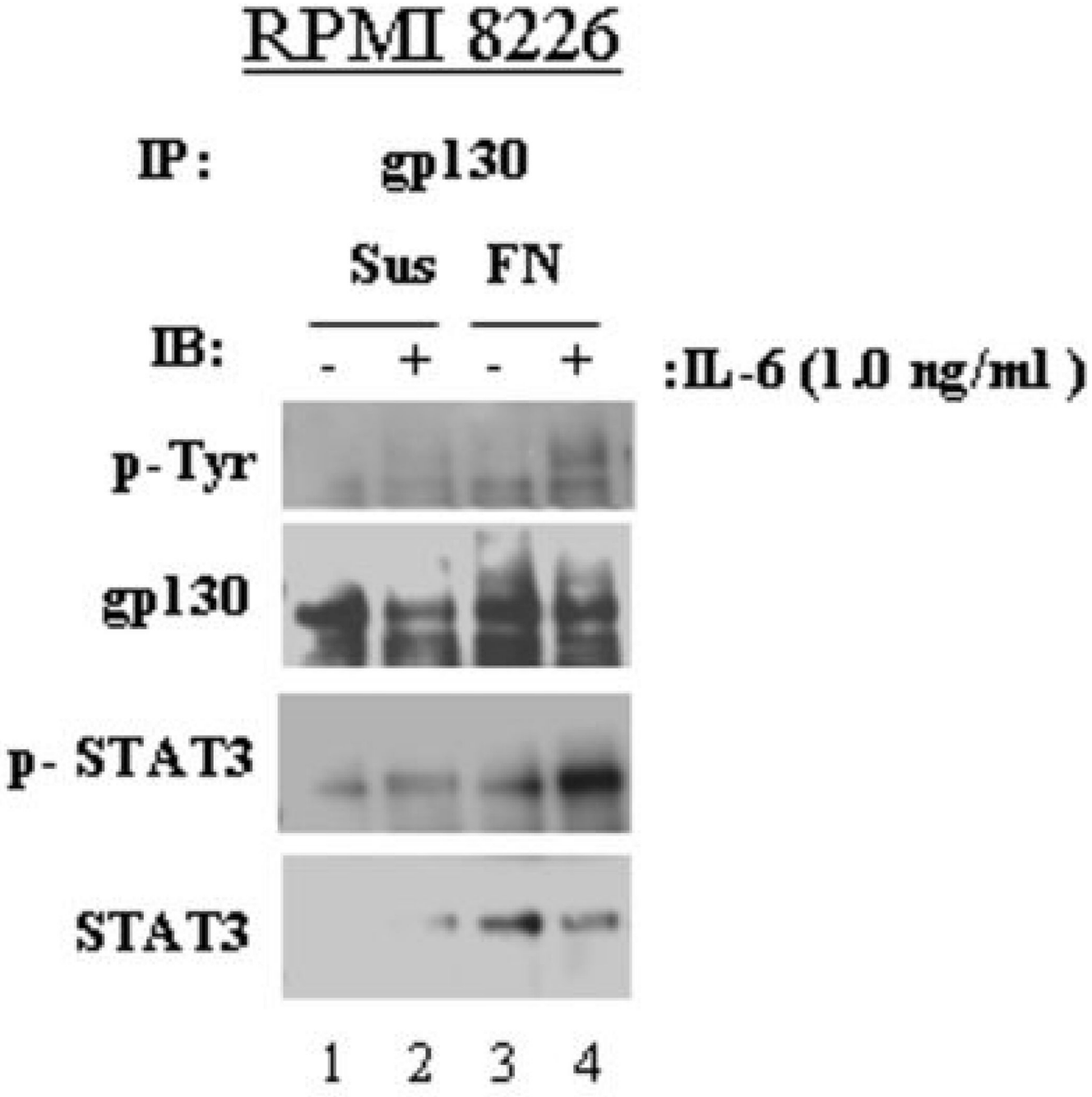

Figure 5. Adhesion of MM cells to FN facilitates recruitment of STAT3 to gp130 independently of IL-6 stimulation

Examination of receptor proximal events via immunoprecipitation of gp130 revealed enhanced tyrosine phosphorylation of the gp130/Jak family complexes in RPMI 8226 cells stimulated with IL-6 while adhered to FN (phosphotyrosine at 125–130 kDa). Increased levels of phosphorylated STAT3 were also observed in association with gp130 under co-stimulatory conditions relative to IL-6 or FN adhesion alone. STAT3 (non-phosphorylated) was also observed in association with gp130 in cells adhered to FN in the absence of IL-6 stimulation (lane 1 vs. 3).

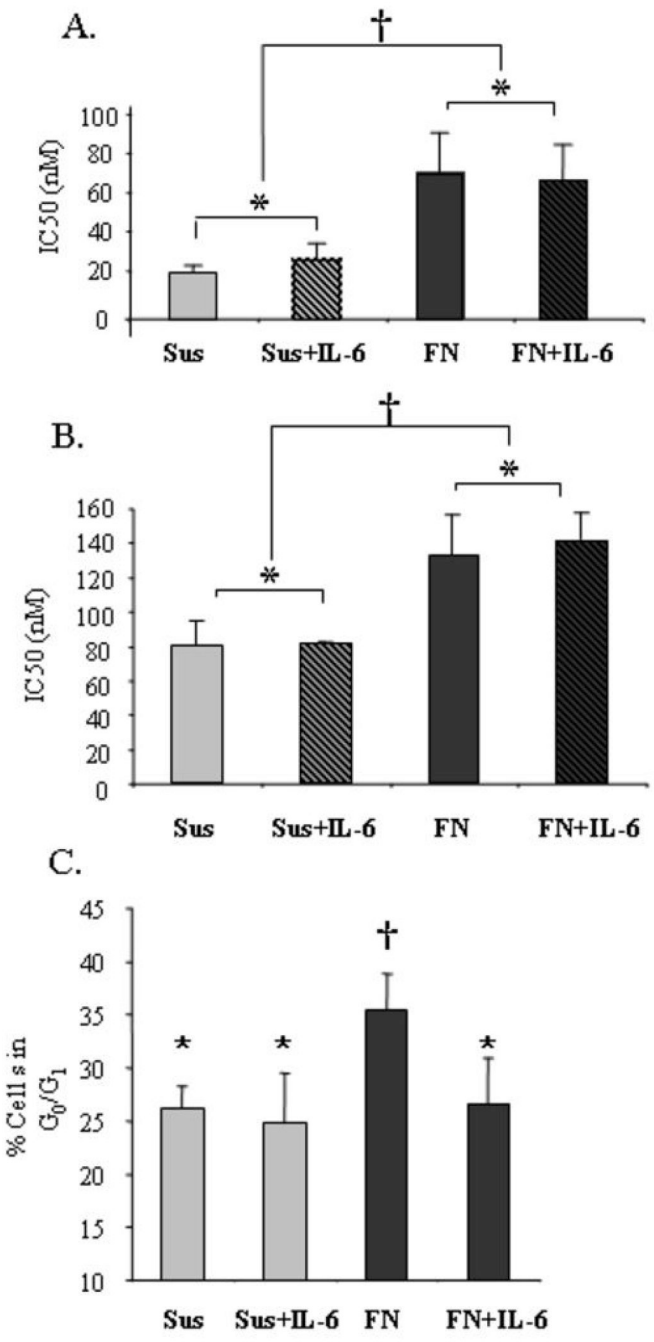

Figure 6. CAM-DR is maintained despite a reversal of the FN-mediated cell cycle arrest in co-stimulated MM cells

CAM-DR has been shown to protect cells from the cytotoxic agents mitoxantrone and doxorubicin. Further, IL-6-mediated STAT3 signaling events also elicit cytoprotective effects. Together, these data suggest that the observed cooperation between FN adhesion and IL-6 may facilitate a greater protection than either effector alone. As demonstrated by 96 hr MTT analysis of MM cell survival, treatment of FN-adhered cells with IL-6 demonstrated no survival advantage in response to **A)** mitoxantrone or **B)** doxorubicin as compared to that mediated by FN alone. **C)** FCM analysis using BrdU/PI incorporation assays demonstrated that FN adhesion facilitates an accumulation of cells in the G_0/G_1 phase of the cell cycle by 24 hrs. As shown,

treatment of cells adhered to FN with IL-6 (1.0 ng/ml) reversed the adhesion-mediated arrest. Asterisks (*) and daggers (†) denote no statistical difference and statistical difference, respectively, as determined by analysis of variance (ANOVA).